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## Characterisation and cardiac directed differentiation of canine adult cardiac stem cells

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### ABSTRACT

This study describes the isolation and characterisation of adult canine cardiac stem cells, and explores their ability to differentiate into cardiac myocytes. Direct comparisons are also made with available human data. Atrial cardiac explants were taken from dogs post-mortem and cultured to isolate adult stem cells. Cells were able to survive successive passages in serum-free media, were able to form cardiospheres, and under controlled culture conditions were capable of clonal expansion, demonstrating their ability for self-renewal. Characterisation of these cells demonstrated the following marker profile: c-kit, GATA 4 and flk-1 positive; cardiac troponin T and NKx2.5 low. Cardiac lineage directed differentiation was performed based on the published literature. Gene expression studies demonstrated that cardiac directed differentiation was partially achieved, with up-regulation of cardiac troponin T and NKx2.5, and down-regulation of c-kit and endothelial lineage markers. However the cells did not express the ryanodine receptor or  $\beta_1$ -adrenergic receptors and did not contract spontaneously.

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### Introduction

Recently the heart has been shown to possess a natural population of stem-like cells (Anversa and Kajstura, 1998; Beltrami et al., 2001; Nadal-Ginard et al., 2003). Messina et al. (2004) identified and characterised a cell population from both the mouse and human heart with properties of adult cardiac stem cells (CSCs). The isolated cells grew as self-adherent clusters, which they termed 'cardiospheres'. These cells were capable of self-renewal and in the case of murine cells could spontaneously differentiate in vitro and in vivo into cardiomyocytes, endothelial cells and smooth muscle cells. The human cells were also shown to differentiate but only following co-culture with rat neonatal cardiomyocytes (Messina et al., 2004). Other studies have also shown evidence for cardiac self-renewal; Bergmann et al. (2009) used radio-carbon dating and ageing cardiac cells from human surgical patients, identifying cells that had originated post-partum.

Although the majority of experimental evidence clearly shows that adult stem cells (ASCs) are present in the heart, our knowledge of these cells in terms of their characteristics and marker expression is limited and the conditions that drive their differentiation are unclear. The variety of species studied, the variation in methods used and the range of markers examined are all confounding

factors. Several studies have shown that cells from experimental animal models such as mice appear to differentiate spontaneously using specialised differentiation media. Conversely, experiments using porcine tissue or human tissue, which are often reliant upon biopsy specimens from patients with cardiac disease, appear to require co-culture with other cell types such as the rat neonatal cardiomyocyte (Beltrami et al., 2003; Messina et al., 2004; Smith et al., 2007).

Further confounding the field, and in contrast to previous studies, Andersen et al. (2009) have recently questioned the ability to isolate adult CSCs from murine and rat tissues. Interestingly, in this particular study, neonatal tissues were utilised and spontaneous beating was seen in some of the explants in culture. Furthermore protocols for isolation of CSCs were adapted from standard techniques. This highlights the difficulty in drawing inferences from published literature and identifies the requirement for studies in the specific species of interest.

In dogs, cardiac disease causes significant morbidity and mortality, contributing to over 50% of mortalities in some breeds such as the Cavalier King Charles spaniel (Bonnett et al., 2005). Typically the dog is predisposed to either endocardiosis or dilated cardiomyopathy. Although an increasing number of treatment modalities are available these are all entirely supportive and not curative. Evaluation of the possibilities for regenerative medicine in the treatment of cardiac disease requires characterisation of the endogenous stem cell population. In addition the stem cells may

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play a role in disease pathogenesis in conditions such as cardiomyopathy. Although two studies have looked specifically at canine CSCs (Bartosh et al., 2008; Linke et al., 2005), they provide limited information in terms of characterisation or requirements for differentiation.

The present project aimed to address three specific and fundamental questions: (1) Can adult CSCs be derived from the canine heart? (2) How do these cells compare with stem cells from other species in terms of behaviour in culture and expression of markers? and (3) Can these cells undergo cardiac directed differentiation?

## Materials and methods

### Tissue preparation and isolation of cardiac stem cells

Full thickness canine cardiac tissue was harvested from the right and left atria and ventricles immediately post-mortem. Dogs were euthanased for clinical reasons and tissues harvested with owner consent.

The tissue was dissected and digested with 0.2% trypsin (Invitrogen) and 0.1% collagenase IV (Invitrogen). Explants were placed into fibronectin (Costar) coated T25 flasks with complete explant medium (CEM) which comprised Iscove's Modified Dulbecco's Medium (IMDM; Invitrogen) with 10% fetal calf serum (Invitrogen), 100 U/mL penicillin G and 100 µg/mL streptomycin (both Invitrogen), 2 mmol/L L-glutamine (Gibco), 0.1 mmol/L 2-mercaptoethanol (2-ME; Sigma) and were maintained at 37 °C in 5% CO<sub>2</sub>.

Explants adhered to the flasks generating a fibroblast-like layer with phase-bright (PB) non-adherent cells floating above. Cells ( $5 \times 10^4$ ) were seeded onto 24-well plates in 600 µL basic cardiosphere growing serum-free media: 35% IMDM; 65% Dulbecco's Modified Eagle's Medium (DMEM) – Ham F-12 mix (Invitrogen) with 2% 50X B27 (Invitrogen); 50 mM 2-ME; 10 ng/mL epidermal growth factor (EGF, Peprotech); 20 ng/mL basic fibroblast growth factor (bFGF, Peprotech); 40 nmol/L cardiotrophin-1 (Invitrogen); 40 nmol/L bovine thrombin (Sigma); 100 U/mL penicillin G; 100 µg/mL streptomycin; 2 mmol/L L-glutamine. Cardiotrophin (2.5 ng/mL), EGF (1.5 ng/mL) and bFGF (1.5 ng/mL) were added every 2–3 days.

### Clonal expansion analysis

Plates (48-well) coated with 0.1% gelatin type A (Sigma) were seeded with  $1 \times 10^4$  cells/well in 500 µL of cardiosphere media. Individual cells adherent to the gelatin were visible 12 h later. Plates were incubated at 37 °C in 5% CO<sub>2</sub>. Cells were fed every 2 days by half media changes. After 6 days in culture large spheres could be seen arising from the individual cells, indicating clonal expansion.

### RNA extraction and RT-PCR analysis

Total RNA from stem cells and control tissues was isolated using the RNeasy Mini Kit (Qiagen), cleaned up using RNase-free DNase kit (Qiagen), and quantified using the Thermo Scientific Nanodrop (Thermo Scientific).

For semi-quantitative RT-PCR, RNA was reverse-transcribed using the Omniscript Reverse Transcription kit (Qiagen). PCR was performed using GoTaq PCR Core Systems kit (Promega). Primer pairs were designed based on published canine sequences (NCBI) and the Primer3 software<sup>1</sup> (Table 1). NKx2.5 primers were taken from Vaags et al. (2009). GAPDH was multiplexed creating an internal positive control. Bands of corresponding sizes were sequenced and compared to the canine genome (NCBI).

For quantitative RT-PCR RNA was reverse-transcribed using Transcriptor High Fidelity cDNA Synthesis kit (Roche). Reactions were run using the Lightcycler 480 Probes Master kit. Data were normalised to 18s RNA (Applied Biosystems). A standard PCR was also performed using individual primers and products run on a 3% agarose gel to confirm specificity of primers.

### Immunofluorescence analysis

Cardiosphere cultures were incubated with rabbit polyclonal anti-c-kit antibody (Dako, Cat No. A4502; dilution: 1:100) previously validated in the dog (Morini et al., 2004), and an anti-flk-1 antibody (Abcam, Ab2349; dilution: 1:100). Secondary Alexafluor 488 conjugated goat polyclonal anti-rabbit IgG (Invitrogen) diluted at 1:500 was used. Nuclear counterstaining was performed using DAPI mount (Vectastain).

### Differentiation of cardiac stem cells

CSCs/well ( $2 \times 10^4$ ) were placed into a 48-well plate with 0.1% gelatin coating. Four differentiation protocols were followed in triplicate following 5 days of stem cell colony establishment. Protocols 1 and 2 were based on Smits et al. (2009) and Protocols 3 and 4 followed Oh et al. (2003). Triplicates of control CSCs maintained in cardiosphere media were run concurrently. Individual protocols were initiated as follows:

Protocol 1: Cells were placed in differentiation media for 24 h (235 mL of IMDM and 235 mL of Ham's F12 nutrient mixture with GlutaMAX-I, 10 mL fetal calf serum,  $1 \times (5 \text{ mL})$  MEM non-essential amino acids,  $1 \times (5 \text{ mL})$  insulin-transferrin). This was followed by 3 days in differentiation media plus 5 µM 5-Azacytidine (5' AZA Sigma). Then, the cells were returned into standard differentiation media, refreshed every 2 days with  $1 \times$  ascorbic acid (Sigma) and 1 ng/mL of TGF-β (Peprotech) every 4 days.

Protocol 2: As above but excluding addition of TGF-β.

Protocol 3: Cells were cultured in M199 media (Invitrogen) plus 10% fetal calf serum. Media was replaced daily for 3 days and then removed and replaced with M199 media plus 2% fetal calf serum with 3 µM 5-AZA. This media was replaced every 3 days.

Protocol 4: As for protocol 3 excluding addition of 5-AZA.

All protocols were continued for 22 days.

## Results

### Cardiac stem cell culture

CSCs were cultured from a total of 25 dogs. Cardiac explants grew an adherent fibroblast layer overlaid with spherical PB cells either singly or in grape-like clusters (Fig. 1A and B). Subjectively, atrial explants consistently produced more PB cells compared to the ventricles and were therefore used for all data in this study.

**Table 1**

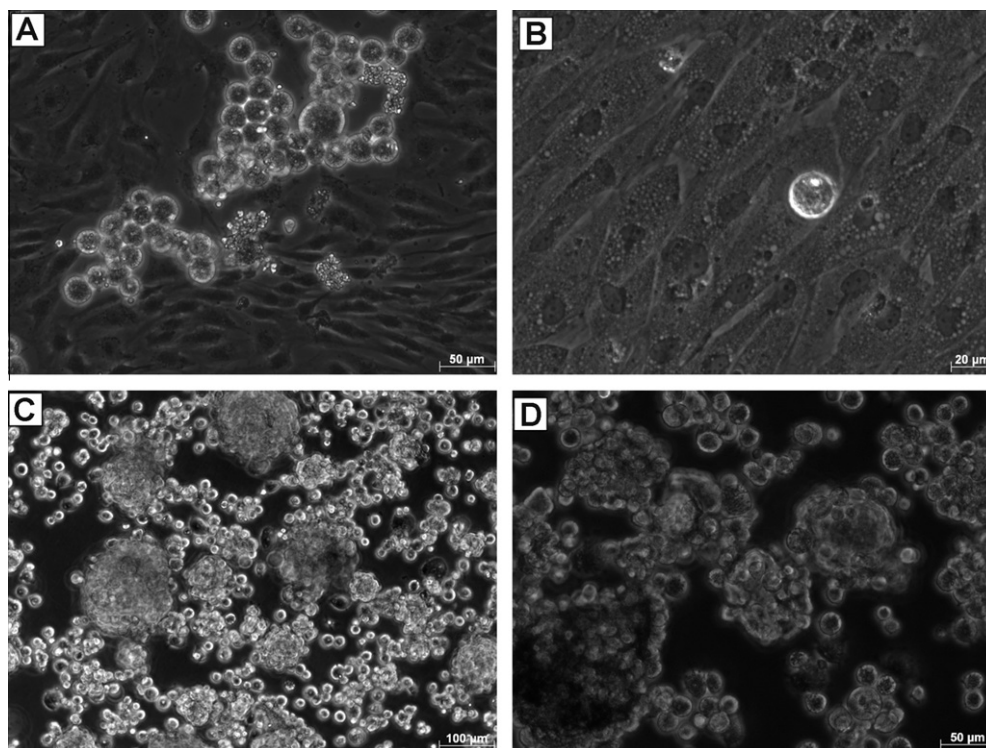
Primer pairs designed for marker expression profiles, including markers for stem cells, cardiac lineage stem cells and differentiated cells. CTT, cardiac troponin T; beta 1 ad, β<sub>1</sub>-adrenergic receptor; cardiac RyR, cardiac ryanodine receptor; CTI, cardiac troponin I; VEGF, vascular endothelial growth factor; VWF, von Willebrand factor; SMA, smooth muscle actin.

Marker	Sequence 5'–3'	Predicted product size
C-kit	F: ATA TCC CAA ACC GGA GCA C R: TCA CCG AAG AAT TGA CAT CG	193
Islet 1	F: GGT TTC TCC GGA TTT GGA AT R: CAC GAA GTC GTT CTT GCT GA	183
NKx2.5	F: CCA AGG ACC CTC GAG CTG A R: CGA CAG ATA CCG CTG CTG CT	185
GATA 4	F: CAA GAT GAA TGG CAT CAA CC R: GGT TTG AAT CCC CTC TTT CC	216
Flk 1	F: CCA CCC AGA CTC AGC ATA CA R: CAC TTT TGG AAT CGT GAG CA	188
CTT	F: GAA GGA CCT GAA CGA ACT R: CCT CCT GIT CTC CTC CTC CT	210
Beta 1 ad	F: GAG TAC GGC TCC TTC CTG TG R: AGC ACT TGG GGT CGT TGT AG	274
Cardiac RyR	F: AAG CGA AGC AGC CCA AGG GT R: TCA GCA AAG TGT GCC GCG CT	888
CTI	F: TCT GCG CAT GGC GGA TGA GA R: TTG CGG CCC TCC ATT CCA CT	624
VEGF	F: TTC CTG CAG CAT AGC AAA TG R: AGG GAG GCT CCT TCT TCC AG	293 (239,311)
VWF	F: CTG GGA GAA GAG AGT CAC GG R: GTG GAT GGA GTA CAC GGC TT	235
SMA	F: GGG GAT GGG ACA AAA GGA CA R: GCC ACG TAG CAG AGC TTC TCC TTG A	525
Glomulin	F: TTG AAG AGC CCT CTG GAA AA R: ACT CCA AAG GGT GGA CAA TG	131
GAPDH	F: CAT CAA CGG GAA GTC CAT CT R: GTG GAA GCA GGG ATG ATG TT	428

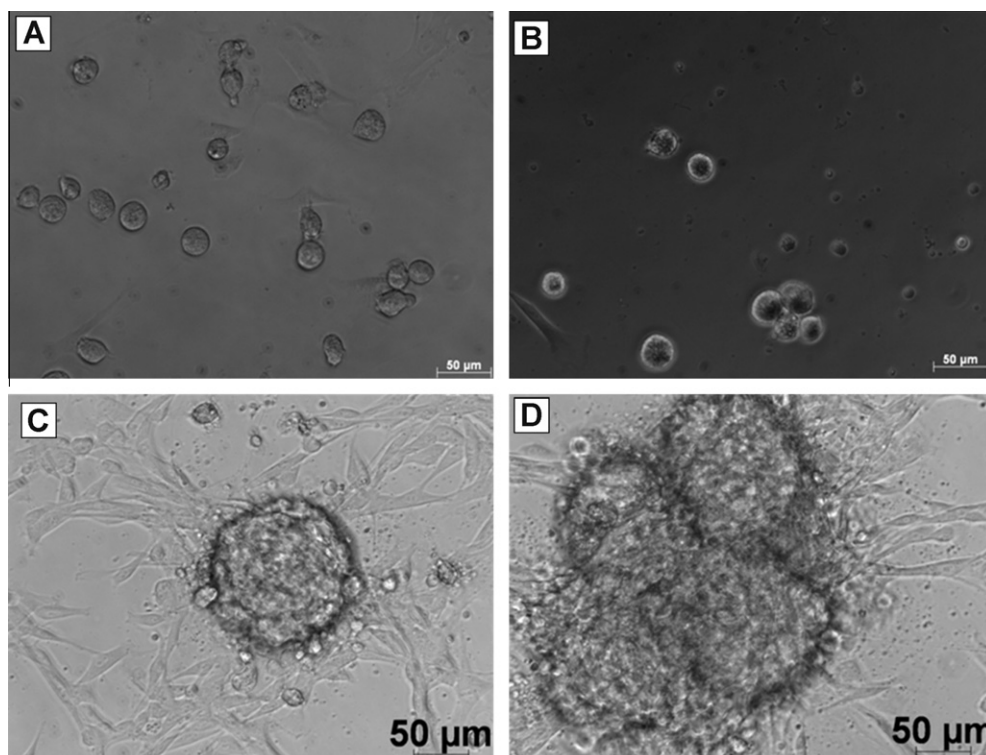
<sup>1</sup> <http://primer3.sourceforge.net/>.

CSCs were placed into serum-free media and over 2–3 weeks formed cardiospheres, which continued to increase in size and number, and could be harvested and passed (Fig. 1C and D).

When CSCs were plated at low density, clonal expansion was demonstrated. Single cells adhered to gelatin within 12 h (Fig. 2A and B) and developed large spherical colonies over 5–7 days



**Fig. 1.** In vitro culture of cardiac stem cells (CSCs). Large spherical, phase-bright cells migrated from the explant overlying a fibroblast-like adherent layer (A), magnification 200 $\times$ . These cells were loosely attached to the fibroblast-like layer or free floating (B), magnification 400 $\times$ . These cells resembled cardiospheres as previously described (C), magnification 100 $\times$ , and (D), magnification 200 $\times$ .



**Fig. 2.** Clonal expansion. Cardiac stem cells were plated at low density to allow visualisation of single cells (A and B), magnification 200 $\times$ . Large spheres grew out from the individual cells indicating clonal expansion (C and D), magnification 200 $\times$ .

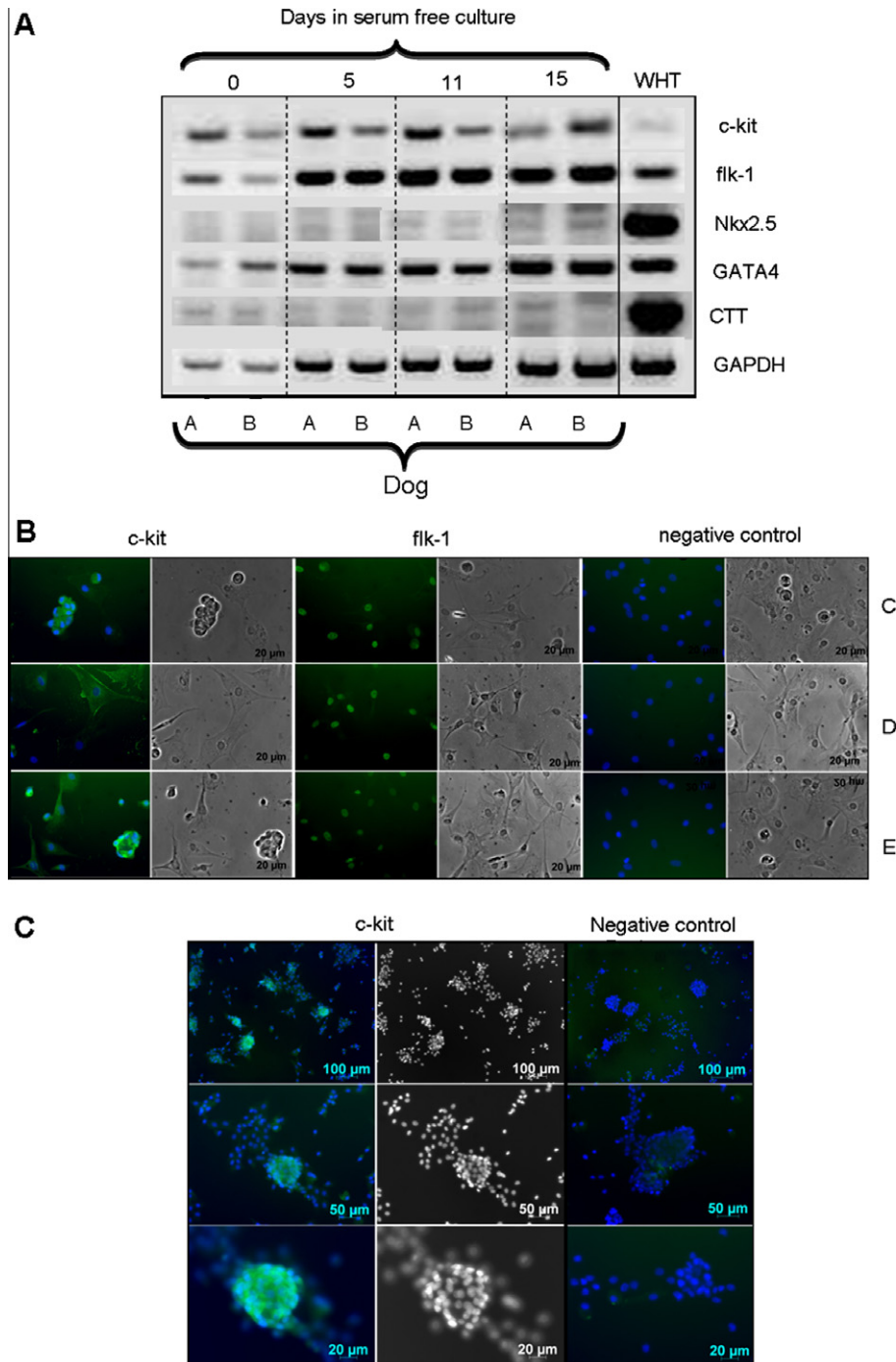


indicative of clonal expansion (Fig. 2C and D). These spheres could be passaged, creating more spheres. At passage, a large central cell could be seen, surrounded by cells of varying sizes, suggestive of asymmetric division. CSCs plated as single cells did not survive culture suggesting that the CSCs required the presence of other CSCs to trigger survival.

#### Cardiac stem cell analysis

CSC populations were characterised using RT-PCR for the following markers (Table 1); stem cell markers (c-kit and islet 1)

(Beltrami et al., 2003; Cai et al., 2003; Goumans et al., 2007; Kubo et al., 2008; Laugwitz et al., 2005; Moretti et al., 2006; Tallini et al., 2009), early cardiac differentiation markers (NKx2.5, GATA 4 and flk-1) (Beltrami et al., 2003; Goumans et al., 2007; Laugwitz et al., 2005; Lints et al., 1993; Matsuura et al., 2004; Wu et al., 2006), cardiomyocyte markers (cardiac troponin T [CTT],  $\beta_1$  adrenergic receptor [Beta 1 Ad], ryanodine receptor [RyR], and cardiac troponin I [CTI]), endothelial cell markers (vascular endothelial growth factor [VEGF] and von Willebrand's Factor [vWF]), and smooth muscle markers (smooth muscle actin [SMA] and glomulin).



**Fig. 3.** Characterisation of cardiac stem cells (CSC). (A) RT-PCR analysis of CSC expression following extended culture in cardiosphere media. Two representative dogs are shown (A and B labelled beneath); culture in serum-free media in days labelled above. (B) CSCs express c-kit and flk-1 (dogs C–E), magnification 400 $\times$ . Greyscale images show cell morphology. (C) Fully developed cardiospheres express c-kit (magnifications 100 $\times$ , 200 $\times$ , 400 $\times$ ; top, central and bottom rows, respectively). DAPI was used to counterstain nuclei (greyscale images from the central column). Negative controls were performed by labelling with secondary antibody only.

Marker expression profiles of CSCs were analysed over a 15-day time course. Markers were compared to whole heart tissue, with GAPDH as a multiplexed positive control (Fig. 3A). C-kit was expressed in all canine CSCs with some variation in levels between dogs and a lower level expression was detected in whole heart. Islet 1 expression was not detected. Flk-1 expression was detected at all time points with a lower expression in whole heart. Both NKx2.5 and CTT were expressed strongly only in whole heart.

GATA 4 was clearly expressed in all cell samples and whole heart. The patterns of expression showed little variation over the 15 days. Using IFA CSCs stained positively for c-kit (cell surface and cytoplasmic distribution) and flk-1 (nuclear distribution) (Fig. 3B).

Differentiation of cardiac stem cells towards cardiac lineages

CSCs were exposed to four differentiation protocols (Fig. 4). A control culture of stem cells maintained in stem cell media was run concurrently, which formed cardiospheres. At 12 h all wells looked morphologically similar (Fig. 5A), with individual stem cells adhering to the gelatin layer. The cells began to form a confluent adherent layer 3 days after protocol initiation, whereas control cells in cardiosphere media formed individual adherent spheres. Morphologically cells exposed to differentiation protocols (protocols 1 and 3) appeared to line up, unlike the cells exposed to the modified protocols aimed at limited or no differentiation (protocols 2 and 4). After 3 weeks, the control stem cell line was still forming spheres.

Gene expression profiles for each protocol were analysed and compared to a standard CSCs and whole heart expression pattern. CSCs following protocols 1 and 2 appeared to have similar expression patterns to each other: low c-kit, NKx2.5, vWF, and CTT; no expression of islet 1, CTI, RyR and Beta 1 Ad expression, and a relatively high GATA 4, flk-1, VEGF, SMA and glomulin expression. Those following protocols 3 and 4 demonstrated differences in expression. When protocols 3 and 4 were compared to the standard controls (Fig. 5C; dogs D and E), (1) c-kit and vWF were down-regulated in protocol 3 compared to protocol 4; (2) NKx2.5 appeared up-regulated in protocol 3 compared to protocol 4; (3)

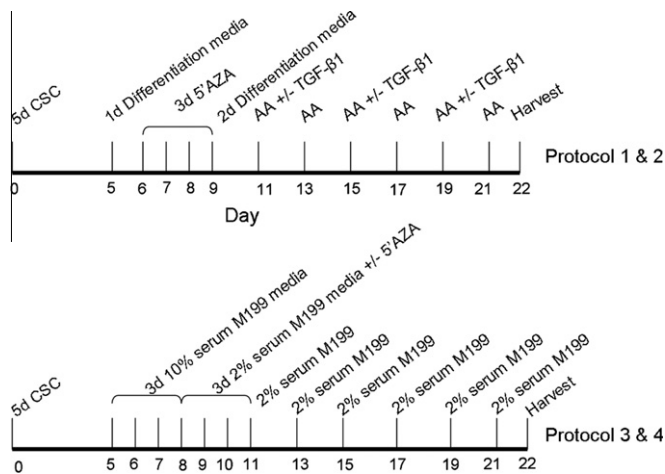


Fig. 4. Differentiation of cardiac stem cells. Four protocols were designed: 1 and 2 were based on Smits et al. (2009), 3 and 4 on Oh et al. (2003); these included the standard and a modified differentiation protocol adapted from each.

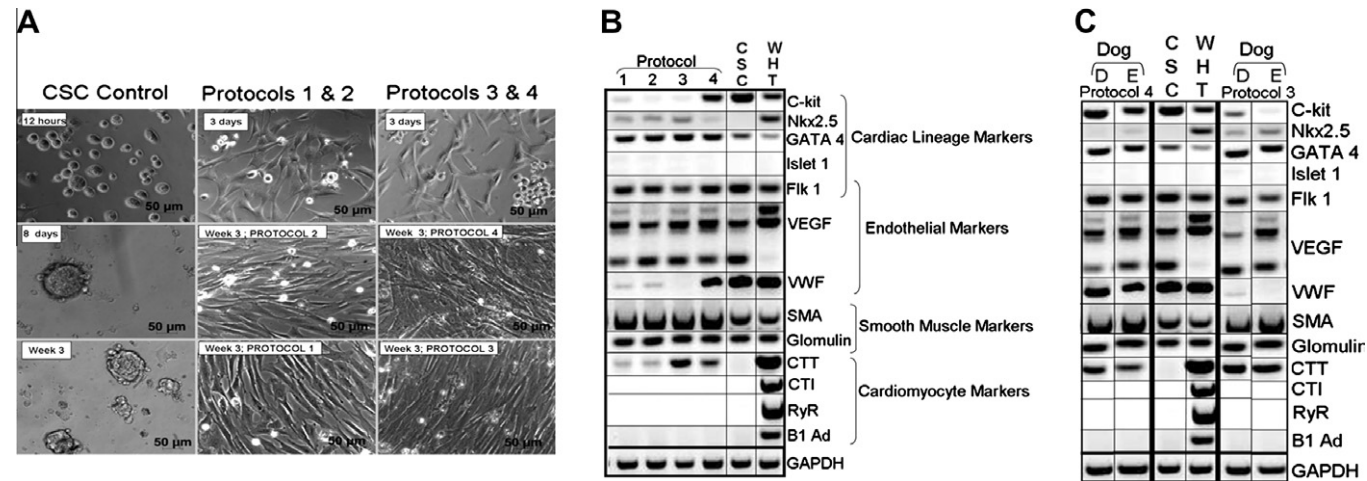


Fig. 5. Analysis of cardiac stem cell differentiation. (A) Time course of morphological changes upon induced cardiac differentiation (protocols 1–4); a control culture of stem cells maintained in serum free culture conditions (CSC) (magnification 200×). (B and C) RT-PCR of control culture (CSC), whole heart tissue (WHT) and stem cells after 22 days of cardiac differentiation. (B) Stem cells extracted from dog E under each protocol (1–4). (C) Stem cells from dogs D and E under differentiation protocols 3 and 4. Markers were designed to analyse cardiac development lineage and endothelial, smooth muscle, and functional cardiomyocyte lineage as indicated in the text.

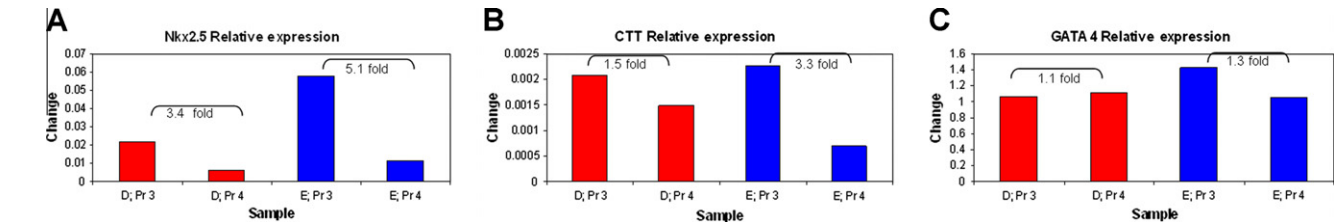


Fig. 6. Relative expression changes following cardiac stem cell differentiation. Quantitative RT-PCR for NKx2.5 and GATA 4 and cardiac troponin T (CTT) of stem cells from dogs D and E differentiated under protocols (Pr) 3 and 4. Results were standardised to 18s ribosomal RNA controls and compared to expression levels from whole heart.

GATA 4 expression remained high following both protocols; (4) no islet 1 expression was seen; (5) flk-1, VEGF, smooth muscle actin and glomulin expression remained at high levels across both protocols; (6) CTT expression was up-regulated following protocol 3 with a larger increase seen in dog E compared to dog D cells. In all four protocols, there was no expression of the cardiac functional protein genes CTI, RyR and Beta 1 Ad.

Quantitative analysis of NKx2.5, GATA 4 and CTT expression was examined compared to whole heart tissue expression. A 3.4- and 5.1-fold (dogs D and E, respectively) up-regulation in NKx2.5 expression was observed in the cells under protocol 3 compared to protocol 4. However, GATA 4 appeared unchanged between the two protocols. CTT had an approximate 1.5-fold up-regulation in dog D and a 3.3-fold up-regulation in dog E (Fig. 6) using protocol 3 compared to protocol 4.

## Discussion

In this study, we have isolated cells from the canine heart, which demonstrate phenotypic and genotypic characteristics consistent with ASCs. The culture techniques proved reliable in reproducing directly comparative cardiosphere populations from multiple dogs of different ages and sex and these cardiospheres behaved similarly to those described for other species (Messina et al., 2004; Smith et al., 2007). Canine CSCs could be grown in serum free conditions, a standard technique for CSCs (Messina et al., 2004). In addition, cells were capable of clonal expansion, a critical characteristic of stem cells.

Canine CSCs expressed key markers consistent with CSCs and did not express cardiac structural and functional proteins. C-kit was expressed at CSC harvest and at the cardiosphere stage and was demonstrated at both the transcriptional and translational levels. C-kit is an accepted CSC marker and has been used in other species, in particular humans. C-kit positive stem cells have been found to be able to differentiate into cardiomyocytes (Beltrami et al., 2003; Goumans et al., 2007; Kubo et al., 2008; Tallini et al., 2009). C-kit in combination with NKx2.5 has been used to define a CSC population able to differentiate into cardiomyocytes and smooth muscle (Lints et al., 1993; Wu et al., 2006).

Flk-1 was expressed at both the RNA and protein levels with the protein in a nuclear location, indicating an active form. Flk-1 has been shown to be expressed in some, but not all CSC populations. Bearzi et al. (2007) described a human CSC population positive for c-kit and negative for flk-1. The same author also described a c-kit positive, flk-1 positive population which behaved as a coronary vascular progenitor cell (Bearzi et al., 2009). Yang et al. (2008) isolated flk-1 positive, c-kit negative stem cell populations from the human heart which gave rise to colonies which contained all three cardiac lineages. This interesting variation in c-kit and flk-1 expression may represent a heterogeneous stem cell population or different developmental stages.

GATA 4 expression was seen in both CSCs and whole heart, as previously described (Goumans et al., 2007). GATA 4, an early transcription factor expressed in the developing heart, is expressed abundantly in CSCs and cardiac myocytes during their lifespan and is thought to be cardioprotective, preventing post-infarction remodelling (Beltrami et al., 2007; Goumans et al., 2007; Heikinheimo et al., 1994; Laugwitz et al., 2005; Matsuura et al., 2004; Rysa et al., 2010). NKx2.5, an early expressed homeobox gene, was expressed in whole heart tissue but not in the canine CSCs (Martin et al., 2004). NKx2.5 has commonly but not exclusively been found to be expressed in CSCs (Beltrami et al., 2003; Goumans et al., 2007; Laugwitz et al., 2005; Oh et al., 2003). Canine CSCs were positive for the endothelial marker vWF, an important finding in CSCs

and one not described in other tissue specific ASC populations (Hu et al., 2003; Kattman et al., 2006; Welikson et al., 2007).

Finally, in agreement with CSC populations isolated from other species (Bearzi et al., 2007; Beltrami et al., 2003; Goumans et al., 2007; Oh et al., 2003), our cell population was lineage negative with no expression of the cardiac functional genes CTI, RyR and Beta 1 Ad.

CSC differentiation has been described using a variety of culture techniques and pharmacological interventions (Behfar et al., 2002; Goumans et al., 2007; Messina et al., 2004; Oh et al., 2003; Smits et al., 2009; Tallini et al., 2009). We selected two published techniques and designed standard and modified differentiation protocols based upon them (Oh et al., 2003; Smits et al., 2009). Following the differentiation protocols (1 and 3) the cells appeared to be more organised when compared to the modified protocols (2 and 4). This alignment may represent development of an organised cell–cell contact, suggesting a more definitive phenotype.

Protocols 1 and 2 showed little difference in expression patterns, but altered expression from CSCs: c-kit and vWF expression was lost and low levels of CTT and NKx2.5 were seen. Protocols 3 and 4 had more striking results which may suggest that 5'AZA is a critical factor. Following protocol 3, there was loss of expression of c-kit and vWF like in protocols 1 and 2. There was also high expression of NKx2.5 and CTT. This suggests that this protocol was most efficient at driving the CSCs toward a cardiac lineage. Cells following protocol 4 expressed CTT at a lower level and did not lose c-kit and vWF expression or exhibit NKx2.5 expression. No variation in GATA 4 expression was seen in all differentiation protocols, suggesting that its expression is a requirement for these cells during the differentiation process.

SMA and glomulin were highly expressed in the CSCs and may therefore be constitutively expressed. This suggests predetermined smooth muscle differentiation capabilities, which are not affected by the protocols used.

## Conclusions

The canine heart has a reliable and reproducible resident population of adult stem cells. These cells compare favourably with other CSCs and in particular human CSCs. While some degree of differentiation occurred with protocol 3, complete differentiation was not achieved. Cells did not spontaneously contract and expression of key components of the contractile machinery was not detected. Nevertheless this study provides a comprehensive characterisation of canine CSCs and is the basis for further study aiming to optimise the conditions required for differentiation.

## Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people of organisations that could inappropriately influence or bias the content of this paper.

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